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⑤4 Treatment of autoimmune diseases by oral administration of autoantigens.

(57) The invention is directed to a method of treating a T cell-mediated autoimmune disease in animals,

This application is a continuation-in-part of U.S. Patent Application Serial No. 065,734 filed June 24, 1987, and having the title "Treatment of Autoimmune Diseases by Oral Administration of Autoantigens."

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the field of treatment of autoimmune diseases and in particular T cell-mediated or T cell-dependent autoimmune diseases. The present invention teaches the oral or enteral administration of autoantigens, or fragments or analogs thereof, to prophylactically and therapeutically treat these autoimmune diseases.

Brief Description of the Background Art

Autoimmune diseases are caused by an abnormal immune response involving either cells or antibodies directed against normal tissues. A number of strategies have been developed to suppress autoimmune diseases, most notably drugs which nonspecifically suppress the immune response. A method of inducing immunologic tolerance by the oral administration of an antigen to prevent autoimmune responses was first demonstrated by Wells in 1911. Wells, H., *J. Infect. Dis.* 9:147 (1911). The oral induction of unresponsiveness has also been demonstrated for several T-cell dependent antigens. Ngan, J. et al., *J. Immunol.* 120:861 (1978), Gautam, S. et al., *J. Immunol.* 135:2975 (1985), Titus, R. et al., *Int. Arch. Allergy Appl. Immun.* 65:323 (1981). Furthermore, a recent publication describes the oral administration of collagen to suppress collagen-induced arthritis in a mouse model. Nagler-Anderson et al., *Proc. Natl. Acad. Sci. (USA)* 83:7443-7446 (1986).

Scientists have also studied ways to suppress autoimmune diseases in various animal models. Experimental allergic encephalomyelitis (EAE) is a T cell-mediated autoimmune disease directed against myelin basic protein (MBP) and has been studied as a model for multiple sclerosis in several mammalian species. See, Alvord, E. et al., *Experimental Allergic Encephalomyelitis—A Useful Model For Multiple Sclerosis* (Allan R. Uss, New York, 1984). Immunoregulation of EAE is known to be at least partially dependent on suppressor T cells (Ts). It has been shown that Ts are present in rats which have recovered from EAE. Swierkosz, J. et al., *J. Immunol.* 119:1501 (1977). Furthermore, it has been shown that suppressor T cells account for the unresponsiveness to EAE that is exhibited by some mouse strains. Lando, Z. et al., *Nature* 287:551 (1980).

Various methods have been employed to induce antigen-specific suppression of EAE and include immunization with MBP emulsified in incomplete Freund's adjuvant, as shown by Lando, Z. et al., *J. Immunol.* 126:1526 (1981), and intravenous injection of MBP-conjugated lymphoid cells as shown by Sriram, S. et al., *Cell. Immunol.* 75:378 (1983).

Three papers by Alvord et al. are reported in *Annals of Neurology* in Vol. 6 at pp. 461-468, 468-473, and 474-482, respectively (1979). The first and second of these papers disclose the suppression of EAE in monkeys by the parenteral administration of MBP only when administered together with a nonspecific adjunctive factor, e.g., an antibiotic or a steroid. The third report discloses the presence in the cerebrospinal fluid of patients with multiple sclerosis of several proteases that degrade MBP to antigenically active peptide fragments.

Papers by Traugott et al., *J. Neurological Science* 56:65-73 (1982), and Raine et al., *Lab. Investigation* 48:275-84 (1983) disclose that treatment of a strain of guinea pigs suffering from chronic relapsing EAE by parenterally administered MBP alone or in incomplete Freund's adjuvant (IFA) or in combination with a lipid hapten of myelin, namely, galactocerebroside, suppressed the clinical symptoms of EAE.

Furthermore, McKenna et al., *Cell. Immun.* 81:391-402 (1983), discloses that preinjection of rats with guinea pig MBP coupled to syngeneic spleen leukocytes or to syngeneic red blood cells suppressed the subsequent induction of EAE using guinea pig MBP in Freund's complete adjuvant. The degree of suppression correlated positively with the amount of MBP administered.

A report by Strejan et al., *Cell. Immun.* 84:171-184 (1984), discloses that preinjection of rats with guinea pig MBP encapsulated within phosphatidylserin liposomes suppressed the clinical signs and symptoms of EAE that appear in rats injected with guinea pig MBP in complete Freund's adjuvant.

Another paper by McKenna et al., *Cell. Immun.* 88:251-259 (1984), discloses that the suppressive effects of injected guinea pig MBP leukocyte complexes disclosed in their 1983 report was abolished when animals

(according to an English abstract) the parenteral administration of "alkaline myelin protein fragment" and "synthetic encephalitogenic peptide" to guinea pigs with EAE. The animals recovered after administration of "alkaline myelin protein fragment" to said animals sensitized by bovine "alkaline myelin protein fragment" or by "synthetic encephalitogenic peptide."

- 5 A report by Braley-Mullen *et al.*, *Cell. Immun.* 51:408 (1980), and the report by Nagler-Anderson *et al.* noted above, both disclose the suppression of the symptoms of two other experimental autoimmune diseases which are induced by injection of animals with autoantigen lymphocyte conjugates. The Braley-Mullen *et al.* report discloses the suppression of experimental autoimmune thyroiditis in the guinea pig by injection of these animals with thyroglobulin antigen in incomplete Freund's adjuvant. The Nagler-Anderson *et al.* report discloses
10 the suppression of T type II collagen-induced arthritis in the mouse by intragastric administration of soluble, but not denatured, T type II collagens prior to immunization of the animal with T type II collagen in adjuvant.

SUMMARY OF THE INVENTION

- 15 The present invention teaches a method of treating a T cell-mediated or T cell-dependent autoimmune disease in an animal comprising the oral or enteral administration to that animal of autoantigens, fragments of autoantigens, or analogs structurally related to autoantigens specific for the particular autoimmune disease, in an amount effective to treat the autoimmune disease. Both the clinical and histological effects of such diseases are suppressed in a dose-dependent manner. Moreover, the suppression occurs whether the oral or enteral administration occurs before or after onset of the autoimmune disease. Disease is also suppressed by
20 oral or enteral administration of non disease-inducing and disease-inducing fragments of the autoantigen. The oral or enteral administration of autoantigens, therefore, represents an effective, simple method by which an autoimmune disease can be naturally immunoregulated.

BRIEF DESCRIPTION OF THE FIGURES

- Figure 1 is a graph which demonstrates the antigen specificity of orally-induced suppression of the proliferative response in Lewis rats. Animals were fed 500 µg of MBP or BSA on days -7, -5 and -2, then immunized with 100 µg MBP in CFA on day 0. Nine days after immunization lymph nodes were removed and proliferative
30 response to MBP, BSA and PPD (all at 50 µg/ml) determined as described in Example 3. Stimulation index = experimental cpm/control cpm.

Figure 2 is a graph which demonstrates orally induced suppression of adjuvant arthritis, as measured by joint swelling.

Figure 3 is a diagrammatic representation of the protocol for inducing relapsing murine EAE.

- 35 Figure 4 is a bar graph representing the orally-induced suppression of lymphoid cell proliferation in SJL mice. Animals were fed 400 µg MBP 7 times over a 2 week period and immunized with 400 µg MBP in CFA (0.6 mg/ml *M. tuberculosis*). Stimulation index is MBP-induced proliferation divided by background.

- Figure 5 is a graph which demonstrates the antigen specific suppression of popliteal draining lymph node cells (PLNC) responses by spleen and mesenteric lymph node cells (LNC) obtained from myelin basic protein (MBP) fed rats. The results are expressed as percent suppression of PLNC to MBP (circles) as to *Mycobacterium tuberculosis* (squares). Closed circles or closed squares represent the response of spleen cells. Open
40 circles or open squares represent the response of mesenteric lymph node cells.

- Figure 6 is a graph which demonstrates the specific suppression of IgG responses to MBP after oral MBP feeding. Rats were bled at intervals and sera examined for anti-OVA (Figure 6A, open circles) or anti-MBP (Figure 6B, open squares) antibodies. These sera were compared to sera obtained from unfed and challenged animals (closed symbols). Results are expressed as ELISA O.D. 492 levels \pm S.D.
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DESCRIPTION OF THE PREFERRED EMBODIMENTS

- 50 The present invention relates to the treatment of T cell-mediated or T cell-dependent autoimmune diseases by the oral or enteral administration of autoantigens specific for such autoimmune diseases as well as biologically active fragments of the autoantigens, and analogs thereof. The term "treatment" is meant to include both prophylactic measures to prevent such autoimmune diseases as well as the suppression or alleviation of

An "autoantigen" is any substance normally found within an animal that, in an abnormal situation, is no longer recognized as part of the animal itself by the lymphocytes or antibodies of that animal, and is therefore attacked by the immunoregulatory system as though it were a foreign substance. The term "biologically active fragment(s)" of such autoantigens include any partial amino acid sequences thereof that induce the same biological response, i.e., the ability to suppress or eliminate T cell-mediated or T cell-dependent autoimmune response, upon oral or enteral introduction. The term "analog(s)" of such autoantigens include compounds that are so structurally related to these autoantigens that they possess the same biological activity, i.e., the ability to eliminate or suppress T cell-mediated or T cell-dependent autoimmune response, upon oral or enteral introduction. As such, the term includes amino acid sequences which differ from the amino acid sequence of the autoantigen by one or more amino acids (while still retaining substantially equivalent biological activity) as well as chemical compounds which mimic the biological activity of the autoantigens in their ability to suppress or alleviate the symptoms of the disease. Such compounds may consist of tissue from a target organ that is the site of attack in an autoimmune disease.

The primary use of the invention is to treat a large category of diseases that are collectively called autoimmune diseases, including but not limited to multiple sclerosis, myasthenia gravis, rheumatoid arthritis, diabetes mellitus, systemic lupus erythematosus, autoimmune thyroiditis, autoimmune hemolytic anemia, and contact sensitivity disease, which may, for example, be caused by plant matter, such as poison ivy.

Experimental allergic encephalomyelitis (EAE) is a T cell-mediated autoimmune disease directed against myelin basic protein (MBP) and has been studied as a model for multiple sclerosis in several mammalian species. Immunoregulation of EAE is known to be at least partially dependent on suppressor T cells (Ts). It has been shown that Ts are present in rats recovered from EAE and that Ts account for the unresponsiveness to the disease in some mouse strains.

Adjuvant arthritis (AA) is an autoimmune animal model of rheumatoid arthritis which is induced by injecting Mycobacterium tuberculosis in the base of the tail of Lewis rats. Between 10 and 15 days following injection, animals develop a severe, progressive arthritis.

The present invention is based on the discovery and confirmation that the oral or enteral administration of MBP is an effective means of suppressing acute monophasic EAE and that the oral or enteral administration of Mycobacteria tuberculosis is an effective way of suppressing adjuvant arthritis. Orally or enterally induced tolerance is dose-dependent, and both clinical and histological symptoms of the disease are lessened in severity. Because orally or enterally an irrelevant antigen such as bovine serum albumin (BSA) has no effect on susceptibility to EAE, it can be said that the orally or enterally induced tolerance to EAE is specific for MBP, the antigen to which the T cells that mediate the disease are sensitized.

Furthermore, the oral or enteral administration of MBP to rats induces the suppression of immune responses to MBP. For example, lymphoid cell proliferation and the production of anti-MBP antibodies are both decreased. The cells responsible for both the suppression of the disease and suppression of antigen-specific cellular responses in vitro are of T cell origin and are suppressor/cytotoxic CD8+ T lymphocytes.

Thus, as demonstrated below, using the EAE animal model for multiple sclerosis and the animal model for AA, the simple method of administration, orally or enterally, of autoantigens such as MBP, as taught by the invention, is an effective treatment to suppress both the development of autoimmune diseases and certain immune responses to the autoantigens.

By the term "introduction" or "administration" is intended that the autoantigen, its biologically active fragments, or biologically active analogs is introduced into the stomach by way of the mouth through feeding or intragastrically through a stomach tube, i.e., enterally.

In general, the autoantigen, fragment, or analog is introduced, orally or enterally, in an amount of from one to 1000 mg per day, and may be administered in single dose form or multiple dose form. Preferably the autoantigen, fragment, or analog is administered in an amount of from 25 to 850 mg per day. As is understood by one skilled in the art, the exact dosage is a function of the autoantigen, the age, sex, and physical condition of the patient, as well as other concurrent treatments being administered.

Where the autoantigen, fragment, or analog is introduced orally, it may be mixed with other food forms and consumed in solid, semi-solid, suspension, or emulsion form; it may be mixed with pharmaceutically acceptable carriers, flavor enhancers, and the like.

Where the autoantigen, fragment, or analog is administered enterally, it may be introduced in solid, semi-solid, suspension or emulsion form and may be compounded with any of a host of pharmaceutically acceptable carriers, including water, suspending agents, emulsifying agents.

EXPERIMENTAL

Animals:

- 5 Female Lewis rats weighing 150 to 220 g were obtained from Charles River Laboratory, Wilmington, MA, and used in all experiments.

Immunization of Animals:

- 10 Rats were immunized in both hind footpads with 50µg guinea pig MBP emulsified in complete Freund's adjuvant (CFA). In some experiments, 50µg ovalbumin (OVA) (Sigma) was added to the emulsified antigens and injected similarly. EAE was characterized by limb paralysis and scored as follows: 0) no disease; 1) decreased activity, limp tail; 2) mild paralysis, unsteady gait; 3) moderate paraparesis, limbs splayed apart; and 4) tetraplegia.

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Induction of Oral Tolerance:

Rats were fed MBP or bovine serum albumin (BSA) five times at three-day intervals 1 mg in 1 ml PBS using a 23-gauge needle covered with plastic tubing.

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Proliferation Assay:

- Nine days after immunization, the rats were sacrificed and their popliteal lymph nodes were removed. A single cell suspension was prepared by pressing the lymph nodes through a stainless steel mesh. A total of 25 10⁵ lymph node cells (LNC) were cultured with the indicated number of either irradiated (2000 Rads) or intact LNC derived from fed rats in quadruplicate in round-bottomed 96-well plate (Costar). MBP and Mycobacterium tuberculosis (Mt), 50 µg/ml were added to the culture in a volume of 20 µl. The cultures were incubated for 80 hours and were pulsed with 1 µCi [³H] TdR/well for the last 16 hours of culture. The cultures were then harvested on an automatic cell harvester and read on a standard liquid scintillation counter.

- 30 Percent suppression of primed LNC (PLNC) proliferation was calculated by the following formula:

$$\% \text{ Suppression} = 100 \times 1 - \frac{\text{CPM (irradiated LNC from fed rat + PLNC + antigen)}}{\text{CPM (irradiated LNC from untreated rat + PLNC antigen)}}$$

Proliferation Media:

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RPMI (Gibco) was used in all the experiments. The medium was filtered sterile after adding 2 x 10⁻⁵M 2-mercaptoethanol, 1% sodium pyruvate, 1% penicillin and streptomycin, 1% non-essential amino acids, and 1% autologous serum.

- 40 Purification of Different Cell Subsets:

- For depletion of CD3, CD4, and CD8 populations from spleen cells, negative selection was used. Petri dishes were coated overnight at 4°C with 10 ml of 1/1000 goat anti-mouse IgG + IgM antibodies (Tago) in PBS/BSA. The plates were then washed and coated with 3% fetal bovine serum in PBS for 30 min at 20°C and washed again. Lewis LNC were stained with mouse anti-rat monoclonal antibodies (Serotec/Bioproductions) for CD3 (MRC, OX/38), CD4 (W 3/25), or CD8 (OX/8) diluted 1/100 in PBS. The cells were stained for 30 min on ice, washed, and seeded on the precoated petri dishes, 15 million cells/5 ml PBS/plate, at 4°C. The supernatant containing nonadherent cells was aspirated gently 60 minutes later and centrifuged twice before cell examination and counting. This protocol yields cell populations of about 85-95% purity as examined in the fluorescence activated cell sorter by examining membrane immunofluorescence.

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Adoptive Transfer Experiments:

- Donor rats were fed with either MBP or BSA, 1 mg x 5 times, at 3-4 day intervals and sacrificed 4 days after the final feeding. Mesenteric LNC and spleen cells were harvested and injected intraperitoneally either immediately or after activation with concanavalin-A (Con-A), 1.5 µg/ml, in proliferation media for 48 hrs. The number of cells injected for adoptive transfer experiments were as follows: 120 x 10⁶ for whole LNC population,

CD8 depleted LNC. Recipient Lewis rats were immunized with BP/CFA 4 hrs later for the induction of EAE.

Serum Levels of Antibodies:

5 A solid-phase enzyme-linked immuno-absorbent assay (ELISA) was used for determination of antibody titers against MBP and OVA. Microtiter plates were incubated with 0.1 ml per well of 10 µg antigen/ml in doubled distilled water. Plates were incubated for 18 hrs at 25°C. After 3 washes with PBS/tween-20 (Bio-Rad), pH 7.5, plates were incubated with 3% BSA/PBS for 2 hrs at 37°C, washed twice, and 100 µl of diluted serum was added in quadruplicate. The plates were incubated for 2 hrs at 37°C. After three rinses with PBS/tween-20, 10 plates were incubated with 100 µl/well of peroxidase-conjugated goat anti-rat IgG antibody (Tago, USA) diluted 1:1000 in 1% BSA/PBS for 1 hr at 25°C. Color reaction was obtained by exposure to D-phenylenediamine (0.4 mg/ml phosphate) citrate buffer, pH 5.0) containing 30% H₂O₂. The reaction was stopped by adding 0.4N H₂SO₄ and OD 492 nm was read on an ELISA reader.

15 In Vitro Measurement of Antibody Production:

Popliteal and splenic LNC were obtained from fed, naive and challenged rats and seeded at a concentration of 10⁷ cells per ml petri dish either alone or irradiated (2000 Rads) together with other PLNC as indicated. The cultures were maintained in proliferation media, with or without antigen (20 µg/ml), for 3 days in an incubator and then harvested. The diluted supernatants were used to examine the in vitro production and secretion of IgG antibody and were measured for antibody production using an ELISA test as described previously.

Identification of Different Regions of the Myelin Basic Protein Molecule Responsible for Suppression of EAE:

25 Overlapping fragments of the 1-37 region of guinea pig myelin basic protein were synthesized using solid phase peptide technique. Houghten, R., Proc. Natl. Acad. Sci. USA 82:5131-5135 (1985). These fragments were then administered orally in equimolar concentrations to 15 mg of whole myelin basic protein. They were administered on day -7, -5, and -2 prior to immunization. Animals were then challenged with basic protein in Freund's adjuvant according to established procedures and scored.

Demonstration that Oral Route of Administration of a Protein Antigen Determines to Which Fragment There Is an Immune Response:

35 Animals were given whole myelin basic protein, either immunized in the foot pad with Freund's adjuvant or administered orally. Seven to 10 days thereafter, spleen and lymph node cells were removed and restimulated in vitro with different fragments of the myelin basic protein molecule.

EXAMPLES

Example 1

45 The effect of feeding MBP and its peptic fragments on the susceptibility to and severity of acute monophasic EAE was studied in the Lewis rat. Results show that this natural route of tolerance induction suppresses both the development of disease and immune responses to MBP.

To orally induce suppression of EAE, Lewis rats were fed MBP purified from guinea pig brain (Diebler, G., et al., Prep. Biochem. 2:139 (1972)) using a syringe equipped with a 20G ball point needle. Control animals were fed equal amounts of bovine serum albumin (BSA) or saline alone. EAE was induced by immunization with 50 µg MBP emulsified in complete Freund's adjuvant (CFA) containing 200 µg Mycobacterium tuberculosis by injection into the hind footpads. Disease was characterized by hind limb paralysis and incontinence usually between days 12 and 15 after immunization and in all cases rats recovered by day 16. The first series of experiments investigated the effect of number of feedings and dose of MBP on disease expression. Rats were fed various amounts of MBP either once 7 days before (day -7) the day of immunization (day 0) or three times

formalin and 5 ml glacial acetic acid. Slides of paraffin-embedded tissue were prepared from each rat and stained with hematoxylin and eosin. Perivascular inflammatory foci were quantified on coded slides by established procedures (Sobel, R., et al., *J. Immunol.* 132:2393 (1984)). As shown in Table I, feeding rats 500 ug MBP on days -14, -7 and 0 caused a marked decrease in the number of inflammatory lesions in the brain. A moderate decrease was found in animals fed 100 ug and no significant reduction of inflammation was found in rats fed 25 ug MBP.

Example 2

A second series of experiments investigated the effect of feeding MBP prior to or subsequent to immunization with MBP to determine whether the effectiveness of orally-induced suppression is affected by prior exposure to antigen. For these experiments, animals were fed 500 ug MBP three times either before or after active induction of disease (immunization with MBP). The results (Table II) demonstrate that the clinical expression of disease is suppressed whether animals were fed MBP before or after sensitization, the effect being more complete when antigen was fed prior to immunization. However, histologic examination revealed a dramatic reduction of perivascular infiltrates in rats fed MBP either before or after sensitization to MBP. Greater than 60% suppression of disease also occurred when rats were fed three times beginning on days +5 or +7 after immunization (data not shown).

In addition, experiments were performed in which rats were fed 100 ug of MBP at various times, before and after immunization, with MBP. As shown in Table III, disease suppression is seen with single feedings before or after immunization.

Example 3

The effects of oral administration of MBP on cellular and humoral immune responses to MBP were also examined. Proliferative responses to MBP were studied after feeding rats different doses of MBP and following feeding at different times with respect to immunization. Ten days after immunization, rats were sacrificed and single cell suspensions of draining (popliteal) lymph nodes prepared. Cells were cultured in microwells for 4 days, the final 24 hours with ^3H -thymidine added. A volume of 0.2 ml containing 4×10^5 cells in RPMI 1640 containing 2% glutamine, 1% penicillin/streptomycin, 5×10^{-6} M 2-mercapto-ethanol and 5% fetal calf serum was added to each microwell and MBP added at 50 ug/ml. Wells were pulsed with 1 μCi tritiated thymidine, harvested onto fiberglass filters using a multiharvester and counted using standard liquid scintillation techniques.

Results (Tables I and II) demonstrate that feeding MBP causes a pronounced (75-92%) decrease in proliferative responses to MBP. Suppression of proliferation, unlike suppression of disease, occurred at all doses and feeding regimens tested, including feeding after immunization. Orally-induced suppression of the proliferative response to MBP is antigen-specific, as shown in Figure 1. Specifically, feeding MBP does not suppress the proliferative response to purified protein derivative (PPD), an antigen derived from *M. tuberculosis* that

TABLE I

Effect of Feeding Dose on Orally-Induced
Suppression of EAE in Lewis Rats

	<u>Induction of EAE</u>	<u>Immune Response to MBP</u> <u>(percent inhibition)</u>		
	<u>^aClinical Disease</u>	<u>^bHistologic Score</u>	<u>^cProliferation</u>	<u>^dAnti-body</u>
Immunized Controls	19/22	9.2±5.8	-	-
Fed day -7				
25 µg	3/5	ND	75.6±2	ND
100 µg	2/5 [*]	ND	88.9	ND
500 µg	3/10 ^{***}	ND	88.9±2	ND
Fed days -14, -7, 0				
25 µg	3/5	7.2±5.2	82.1	-48±72
100 µg	2/5 [*]	3.2±1.9	80.8±5	14±49
500 µg	0/10 ^{***}	0.2±0.4	87.2±1	66±39

(a) Rats were fed various doses of MBP on the indicated days and immunized with 50 µg MBP in CFA (200 µg *M. tuberculosis*) on day 0. Shown are the number of diseased rats of the total number immunized. Immunized controls were fed BSA or saline.

(b) Rats were sacrificed on day 16 after immunization and brains removed and fixed. Shown are the average number of perivascular inflammatory foci per animal +/- s.d. ND = not determined.

(c) Proliferative response to MBP was measured for draining lymph node cells ten days after rats were immunized. A volume of 0.2 ml containing 4×10^5 cells in RPMI 1640 containing 2% glutamine, 1% penicillin/streptomycin, 5×10^{-5} M 2-mercapto-ethanol and 5% fetal calf serum was added to each microwell and MBP added at 50 µg/ml. Wells were pulsed with 1 µCi tritiated thymidine, harvested, and counted using a multiharvester and counted

percentage inhibition of proliferative response to MBP with respect to the immunized control group. Average stimulation index of the immunized controls (MBP-stimulated CPM/background CPM) was 6.0 (29,888 CPM/4960 CPM).

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(d) Rats were sacrificed on day 16 and blood drawn by cardiac puncture. Sera were diluted 1/15,625 in PBS and anti-MBP antibody levels were determined by ELISA. A volume of 0.1 ml of MBP solution (0.05 mg/ml in PBS) was added per microwell and incubated for 3 h at 37°C. Wells were washed with PBS containing 0.05% Tween (PBST) and blocked overnight at 4°C with 5% BSA in PBS, pH 9.0. After washing wells with PBST, diluted rat sera were added and incubated for 3 h at room temperature and after washing with PBST secondary antibody (peroxidase conjugated goat anti-rat) added for 1 h at room temperature. Substrate was added and the reaction was stopped with 0.1 M NaF. Plates were read at 450 nm on a Titertek multiscan. Abs₄₅₀ was also determined for serum from rats immunized only with CFA and was subtracted from all values as background. Shown is the percentage decrease in antibody level, as measured by absorbance of peroxidase substrate at 450 nm, with respect to immunized controls (Mean absorption at A₄₅₀ of immunized controls with background subtracted was 0.148).

(e) Groups were compared by chi-square analysis with one degree of freedom: * p < .05, ** p < 0.1, *** p < .001.

TABLE II

Effect of Feeding MBP to Rats Before or After
Immunization on the Development of EAE

	<u>Induction of EAE</u>		<u>Immune Response to MBP</u> (percent inhibition)	
	<u>^aClinical Disease</u>	<u>^bHistologic Score</u>	<u>^cProliferation</u>	<u>^dAnti-body</u>
Immunized Controls	23/26	21.6±5.1	-	-
Days fed 500 µg MBP				
-7, -5, -2, +2, +5, +7	0/5 ^{***}	0.2±0.4	ND	34
-7, -5, -2	0/17 ^{***}	0	92.6	15
+2, +5, +7	4/10 ^{**}	1.4±2.3	91.5±3	15

(a) Rats were fed 500 µg MBP on the indicated days and immunized with 50 µg MBP in CFA on day 0. Immunized controls were fed BSA or saline.

(b) See Table I.

(c) See Table I. Average stimulation index of immunized controls was 9.4 (82,247 CPM/8,718 CPM).

(d) See Table I. Mean absorption at A₄₉₂ of immunized controls with

Example 5

Further experiments were conducted to determine the persistence of orally-induced protection against EAE. After feeding on days -7, -5 and -2 with 500 ug MBP rats were immunized at various lengths of time after the last feeding. EAE was completely suppressed in rats for up to four weeks after feeding, and by eight weeks 50% of rats fed MBP were again susceptible to disease. The results are shown in Table IV, which indicates that tolerance to the disease is maintained for at least four weeks after the last feeding, with susceptibility to disease induction becoming apparent at eight weeks following feeding.

TABLE IV

Persistence of Orally Induced Tolerance of Lewis Rats

	<u># Rats Sick/Total</u>
Control	9/14
Fed	
Immunized day 0	0/4
day +7	0/4
day +14	0/4
day +28	0/3
day +56	4/8

Rats were fed 500 µg MBP on days -7, -5, and -2 and immunized on the indicated days with 50 µg MBP in CFA. Control rats (fed BSA) were likewise immunized.

Example 6

It is known that the encephalitogenic region of guinea pig MBP in rats is a specific decapeptide sequence located at residues 75-84, which by itself can induce EAE, whereas other regions of the molecule are non-encephalitogenic (Hashim, G., Myelin: Chemistry and Biology, Alan R. Liss, N.Y. (1980)). Furthermore, for other antigens, it has been reported that distinct suppressor determinants exist at sites different from immunogenic determinants (Yowell, R., et al., Nature 279:70 (1979)). It was therefore investigated whether both encephalitogenic and non-encephalitogenic fragments of MBP could prevent EAE via oral administration. Fragments of guinea pig MBP were generated by limited pepsin digestion and separated by column chromatography (Whitaker, J., et al., J. Biol. Chem. 250:9106: (1975)). The three different fragments were fed to rats, then animals were immunized with whole MBP. It was found that both the disease-inducing (fragment 44-89) and non-encephalitogenic (fragments 1-37 and 90-170) peptides suppressed EAE when fed to rats, the non-encephalitogenic fragments being more effective in suppressing the disease than the encephalitogenic fragment (Table V). Additionally, the fragment which differs from the encephalitogenic sequence (residues 75-84)

TABLE V

The Effect of Feeding Encephalitogenic and
Non-Encephalitogenic Fragments on the Development
of EAE in Lewis Rats

Clinical Incidence of EAE

10	Immunized Controls	19/25
	MBP fragment 1-37 (109 µg)	0/9 ^{a***}
	MBP fragment 44-89 (135 µg)	3/11 ^{**}
15	MBP fragment 90-170 (235 µg)	0/4 ^{**}
	Peptide S79 (30 µg)	1/8 ^{***}
20	Bovine MBP (500 µg)	0/10 ^{***}

25 Lewis rats were fed the indicated amounts of MBP fragments or peptides (equimolar to 500 µg whole guinea pig MBP) on days -7, -5 and -2 and immunized on day 0 with 50 µg guinea pig MBP with CFA. Shown are the number of diseased rats of the total number immunized. (a) Groups were compared to immunized controls by chi-square analysis: ** p < .01, *** p < .001.

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Example 7

Suppression of Adjuvant Induced Arthritis by Feeding Mycobacteria

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Adjuvant arthritis was induced in female Lewis rats by immunization with 0.1 ml of 10 mg/ml of complete Freund's adjuvant in the base of the tail. Animals were fed 2.0 mg of Mycobacteria tuberculosis in phosphate

TABLE VI

Joint swelling (mm) on day 21Control 7.61 \pm 1.4Days Fed Mycobacteria-7, -5, -2 5.61 \pm 1.1*-7, -5, -2, +7, +14 6.07 \pm 0.9*

Joint swelling = thickness of joint on day measured

*p < 0.01 compared to control (representative experiment
of 4 animals/group)

Example 8

An Adoptive Transfer Model of EAE in the SJL Mouse

A workable, reproducible model of adoptive relapsing EAE was established in the SJL mouse. The protocol for this model was adopted from Mokhtarian, et al., Nature, 309:356 (1984). This protocol is depicted graphically in Figure 3. Briefly, donor animals are immunized with an emulsion containing 400 ug of MBP and 30 ug of M. tuberculosis in CFA. Ten days thereafter, draining lymph nodes are removed and cultured with 50 ug/ml of MBP for four days, washed extensively, and $4-6 \times 10^7$ viable cells are injected intravenously into female recipient animals. Animals are scored for clinical EAE using standard scales, and scored pathologically using standard H & E histological analysis (Brown, A., et al., Lab Invest. 45:278 (1981), Lublin, F., et al., J. Immunol. 126:819 (1981), and Bernard, C. et al., Eur. J. Immunol. 16:655 (1976)). Animals are monitored for at least 100 days after transfer so that the number of relapses can be determined.

Example 9

Orally Induced Suppression of Proliferative Responses in SJL Mice

The feeding of 400 ug MBP every other day for two weeks (total of seven separate feedings) prior to immunization with 400 ug MBP in CFA (0.6 mg/ml M. tuberculosis) suppresses the proliferation of lymph node

peritoneally either immediately or after activation with concanavalin-A (Con-A), 1.5 µg/ml, in proliferation media for 48 hrs. The number of cells injected for adoptive transfer experiments were as follows: 120x10⁶ for whole LNC population, either activated or not; 60x10⁶ for CD3 depleted LNC; 80x10⁶ for CD4 depleted population; and 95x10⁶ for CD8 depleted LNC. Recipient Lewis rats were immunized with MBP/CFA 4 hrs later for the induction of EAE. The ability to transfer resistance to development of EAE from fed donor rats to naive syngeneic recipient rats is shown in Table VII. LNC obtained from unfed rats or from bovine serum albumin (BSA) fed donor rats failed to transfer protection against EAE. However, both spleen cells or mesenteric (MES) lymph node cells obtained from MBP fed donors were capable of transferring relative protection against EAE induced in the recipients, demonstrating 50% and 57% suppression of disease, respectively. The mean maximal severity of disease was also reduced markedly in recipients of either spleen cells or mesenteric lymph nodes cells obtained from MBP fed donor rats. These results demonstrate that the oral tolerance to EAE induction is of cellular origin and that the cells responsible for protection are found to be concentrated in both the mesenteric lymph nodes and the spleen.

TABLE VII

Adoptive transfer of protection against EAE using LNC obtained from either fed or untreated donor rats.

Rats Fed with	Donors Source of LNC	EAE in Recipients	
		Incidence	Mean Max. severity
None	SPC	6/7	2.5±0.3
	Mes.LNC	5/5	2.6±0.4
BSA	SPC	4/4	2.4±0.2
	Mes.LNC	5/5	2.6±0.3
MBP	SPC	4/8*	1.6±0.2*
	Mes.LNC	4/7*	1.7±0.2*

Lewis rats were fed with either MBP or BSA five times, 1 mg per feeding at 3 day intervals, or remained untreated. The rats were then sacrificed and their spleens and mesenteric lymph nodes were removed. The LNC were harvested and activated for 48 hours in the presence of Con-A. The lymphoblasts were collected, washed three times, and injected intraperitoneally into naive syngeneic rats. The recipient rats were challenged 4 hours later with MBP/CFA for the induction of EAE. The disease was scored daily from day 10 (*Results are statistically significant, p<0.05).

Example 11

Identification of the Lymph Node Cell Subpopulation which mediates Resistance to EAE

Con-A activated spleen cells (SPC) obtained from MBP fed donor rats were transferred to naive syngeneic rats either before or after depleting either T c lls, helper T lymphocytes (CD4) or suppressor/cytotoxic T lymphocytes (CD8). For depletion of CD3, CD4 and CD8 populations from spleen cells, negative selection was used. Petri dishes were coated overnight at 4°C with 10 ml of 1/1000 goat anti-mouse IgG + IgM antibodies (Tago) in PBS/BSA. The plates were then washed and coated with 3% fetal bovine serum in PBS for 30 min at 20°C and washed again. Lewis LNC were stained with mouse anti-rat monoclonal antibodies (Serotec/Bio-products) for CD3 (MRC, OX/38), CD4 (W3/25) or CD8 (OX/8) diluted 1/100 in PBS. The cells were stained

fore cell examination and counting. This protocol yields cell populations of about 85-95% purity as examined in the fluorescence activated cell sorter by examining membrane immunofluorescence. The results are demonstrated in Table VIII. The results demonstrate that SPC are capable of transferring protection against EAE (50% incidence), whereas T cell depleted SPC lost their ability to protect recipient rats (group 2). Thus, it seems that the spleen cells which are capable of transferring protection are T lymphocytes. However, depletion of CD8 cells (group 4) results in failure of transferring protection, whereas CD4+ depleted SPC showed a significant ability of protecting rats against EAE. Thus, it is evidence that the antigen specific T lymphocytes which are generated after oral administration of MBP and which are mediating resistance to disease induction are of the suppressor/ cytotoxic subset.

TABLE VIII

Adoptive transfer of protection against EAE using depleted population of SPC.

Group	SPC removed from MBP fed donors	EAE in recipient rats	
		Incidence	Mean Max. Severity
1	Whole population	2/4	1.7±0.2*
2	CD3 depleted	6/6	2.6±0.4*
3	CD4 depleted	2/6*	1.2±0.2*
4	CD8 depleted	6/7	2.2±0.3

Donor rats were fed with MBP, and treated as indicated in the legend of Table 1. The Con-A activated SPC were injected into naive

recipient rats either before (group 1) or after depletion of certain subpopulation (groups 2-4). Depletion of CD3, CD4 or CD8 lymphocytes was done by coupling monoclonal IgG antibodies to the SPC and panning. Recipient rats were immunized with MBP/CFA and EAE was recorded from day 10 (*Results are statistically significant, $p < 0.05$).

Example 12

In vitro Suppression of Anti-MBP T Cell Responses by Addition of Lymph Node Cells from MBP Fed Rats

Rats were immunized with MBP/CFA and their primed popliteal draining lymph nodes (PLNC) harvested nine days later. A single cell suspension was prepared by pressing the lymph nodes through a stainless steel mesh. A total of 10^5 LNC were cultured with the indicated number of either irradiated (2000 Rads) or intact LNC derived from fed rats in quadruplicate in round bottomed 96-well plate (Costar). MBP and Mycobacterium tuberculosis, 50 µg/ml were added to the culture in a volume of 20 µl. The cultures were incubated for 80 hrs. and were pulsed with 1µCi [³H] TdR/well for the last 16 hours of culture. The cultures were harvested on an automatic cell harvester and read on a standard liquid scintillation counter.

Percent suppression of primed LNC (PLNC) proliferation was calculated by the following formula:

$$\% \text{ Suppression} = 100 \times 1 - \frac{\text{CPM (irradiated LNC from fed rat + PLNC + antigen)}}{\text{CPM (irradiated LNC from untreated rat + PLNC antigen)}}$$

The PLNC were cultured along with irradiated SPC or mesenteric LNC obtained from either naive or MBP fed rats in the presence of either MBP or Mycobacterium tuberculosis. The LNC obtained from MBP fed donor rats were examined on a different days after last feeding. Results are shown in Figure 5. It is shown that within

the PLNC proliferation to MBP. Antigen specific suppression of PLNC responses was greater using SPC than mesenteric LNC. Suppression is evident from day 5 to day 36 after the last feeding with MBP indicating that the induction of suppression is achieved soon after feeding and it is maintained for a relatively long period of time.

- 5 Thus, it seems that LNC obtained from rats rendered to be tolerized to EAE induction are antigen-specific lymphocytes which are capable of suppressing cellular immune responses only to the antigen used for feeding.

Example 13

10 Suppression of Anti-MBP Responses of PLNC in the Presence of Irradiated SPC and its Subpopulations, Obtained from a MBP Fed Rat

- To examine the subpopulation of SPC responsible for suppression, SPC were obtained from MBP fed rat 20 days after the last feeding, depleted of certain lymphocyte populations, irradiated and mixed with PLNC
 15 obtained from MBP/CFA immunized rat together with MBP. Popliteal and splenic LNC were seeded at a concentration of 10^7 cells per ml petri dish either alone or irradiated (2000 Rads) together with other PLNC as indicated. The cultures were maintained in proliferation media, with or without antigen (20 μ g/ml), for 3 days in an incubator and then harvested. The diluted supernatants were used to examine the *in vitro* production and secretion of IgG antibody and were measured for antibody production using an ELISA test. Microtiter plates
 20 were incubated with 0.1 ml per well of 10 μ g antigen/ml in doubled distilled water. Plates were incubated for 18 hrs. at 25°C. After 3 washes with PBS/tween-20 (Bio-Rad), pH 7.5, plates were incubated with 3% BSA/PBS for 2 hrs. at 37°C, washed twice and a 100 μ l of diluted serum was added in quadruplicate. The plates were incubated for 2 hrs. at 37°C. After three rinses with PBS/tween-20, plates were incubated with 100 μ l/well of peroxidase-conjugated goat anti-rat IgG antibody (Tago, USA) diluted 1:1000 in 1% BSA/PBS for 1 hr. at 25°C.
 25 Color reaction was obtained by exposure to D-phenylenediamine (0.4 mg/ml phosphate citrate buffer, pH 5.0) containing 30% H_2O_2 . The reaction was stopped by adding 0.4N H_2SO_4 and the OD 492 nm was read on an ELISA reader. The results shown in Table IX represents the percent suppression of the antigen proliferation of PLNC in the presence of SPC obtained from MBP fed rats compared to their responses to MBP in the presence of SPC obtained from intact rats. It is demonstrated that SPC obtained from MBP fed rats (group 1) suppresses the responses of PLNC to MBP (70%). Depletion of T cells (group 2) or suppressor/cytotoxic T lymphocytes (group 3) abrogates suppression. However, depletion of helper T lymphocytes (CD4, group 4) enhances the inhibition of the anti-MBP proliferation response of the PLNC. Diluting the CD4 depleted SPC results in decreasing of suppression from 96% (in the 1:1 ratio) to 18% (in the 1:100 ratio 1 SPC:PLNC).
 30

TABLE IX

Suppression of anti-MBP responses of PLNC in the presence of irradiated SPC and its subpopulations, obtained from MBP fed rats.

Group	SPC removed from MBP fed rats	SPC:PLNC ratio	% Suppression of PLNC responses to MBP
1	Whole population	1:1	70
2	CD3 depleted	1:1	-13
3	CD8 depleted	1:1	-30
4	CD4 depleted	1:1	96
	" "	1:10	32
	" "	1:50	35
	" "	1:100	18

Spleens were removed from MBP fed Lewis rats, then cells were harvested, irradiated and seeded along with responder PLNC removed from MBP/CFA immunized syngeneic rats. The SPC were used as untreated cells or depleted of CD3, CD4 or CD8 T lymphocytes using the appropriate monoclonal antibodies for coupling and then panning. Results are expressed as percent suppression of PLNC responses to MBP and are relative to the PLNC responses in the presence of irradiated SPC removed from unfed rats.

Example 14

Humoral Suppression of Anti-MBP IgG Production Induced by Oral Tolerance to MBP

Lewis rats were either fed with MBP or left untreated and then challenged with MBP mixed with ovalbumin (OVA) emulsified in CFA. The rats were then bled at various intervals, and sera was examined for anti-OVA or anti-MBP antibodies. As shown in Figure 6a, the IgG serum levels to OVA were not affected in MBP fed rats, whereas IgG serum levels to MBP were decreased in MBP fed rats (6b).

Example 15

Determination of the Cell Type Responsible for the Suppression of IgG Production In Vitro

Lewis rats were fed with MBP or remained unfed and then were immunized with MBP + OVA/CFA. The PLN were removed 12 days later, and the PLNC were cultured for 3 days in the presence of either MBP or OVA, the supernatants were collected, diluted 1:20 and examined for their IgG contents. As shown in Table X, PLNC, which were obtained from fed rats (group 2) and cultured in vitro with MBP, responded less in terms of IgG production to MBP than to PLNC obtained from unfed rats (group 1, 45% suppression). The

TABLE X

Group	Responder Cells	Modulator Cells	In Vitro Stimu- lation	<u>IgG Levels in Supernatants</u>	
				O.D. 492 Values \pm S.D.	% Suppression of IgG Production
1	Immunized	--	MBP	0.56 \pm 0.06	--
2	MBP Fed and Immunized	--	MBP	0.31 \pm 0.01	45
3	Immunized	MBP Fed and Immunized	MBP	0.36 \pm 0.04	35
4	Immunized	MBP Fed and Immunized CD8 ⁺ depleted	MBP	0.55 \pm 0.04	0
5	Immunized	--	OVA	0.17 \pm 0.03	--
6	MBP Fed and Immunized	--	OVA	0.18 \pm 0.02	0
7	Immunized	MBP Fed and Immunized	OVA	0.21 \pm 0.04	0

Rats were immunized with MBP+OVA and CFA (some 3 days after the fifth feeding of MBP). Twelve days later their PLNC were removed and cultured together with MBP (groups 1-4) or with OVA (groups 5-7) for three days. In some groups, irradiated PLNC obtained from MBP fed and immunized rats were irradiated and cultured along with immunized PLNC in the presence of MBP (group 3) or in the presence of OVA (group 7). The supernatants of these stimulations were collected, diluted and IgG levels determined by ELISA.

Example 15

Identification of the MBP Region which Actively Suppresses EAE using Overlapping Synthetic Polypeptides of MBP

Overlapping fragments of the amino acid 1.37 fragment of guinea pig myelin basic protein were synthesized using solid phase peptid technique. Houghten, R., Proc. Natl. Acad. Sci. USA 82:5131-5135 (1985).

These fragments were then administered orally in equimolar concentrations to 15mg of whole myelin basic protein. The fragments administered were 7, 5, and 2 prior to immunization. Animals were then challenged

control animals became ill with a mortality of 3/6. In animals receiving overlapping peptide fragments, there was decreased mortality using all fragments, except for fragment 1-10. When viewed in terms of disease severity, the region of the molecule between amino acids 5 and 20 shows the most pronounced diminution of disease. These results demonstrate that in the amino acid region 1-37 which itself is a suppressogenic fragment, specific regions of the molecule may be more or less suppressive when administered orally.

TABLE XI

Fragment	Incidence of Disease	<u>EAE Mediated by MBP/CFA</u>	
		Mean Max. Score	Mortality
Control (FBS)	6/6	3.8	3/6
1-10	5/5	3.8	4/5
5-15	4/5	2.1	1/5
11-20	4/5	2.0	0/5
16-25	4/5	2.6	0/5
21-30	5/5	3.0	1/5
26-36	4/6	2.6	1/6
31-37	5/6	3.3	0/6

Overlapping fragments of the 1-37 region of guinea pig myelin basic protein were synthesized using solid phase peptide technique. These fragments were then administered orally in equimolar concentrations to 15mg of whole myelin basic protein. They were administered on day -7, -5, and -2 prior to immunization. Animals were then challenged with basic protein in Freund's adjuvant according to established procedures and scored.

Example 16

Demonstration that Oral Route of Administration of a Protein Antigen Determines to which Fragment there is an Immune Response

Animals were given whole myelin basic protein, either immunized in the foot pad with Freund's adjuvant or administered orally. Seven to 10 days thereafter, spleen and lymph node cells were removed and restimulated *in vitro* with different fragments of the basic protein molecule.

As shown in Table XII, when myelin basic protein is administered peripherally in Freund's adjuvant, the primary response is to the 44-89 encephalitogenic region as measured by proliferation. However, as shown in Table XIII, when administered orally, the primary response is to fragment 1-37, the non-encephalitogenic

TABLE XII

Proliferation to MBP fragments in Lewis rats immunized with whole MBP.

	<u>Counts Per Minute</u>	<u>Stimulation Index</u>
Background	3,292	--
Whole MBP	10,142	3.1
MBP fragment 1-37	3,360	1.0
MBP fragment 44-89	10,054	3.0

Animals were immunized in hind foot pads with 50 μ g MBP in CFA. Ten days later lymph nodes were removed and stimulated in vitro with 10 μ g MBP or equimolar amounts of MBP fragments.

TABLE XIII

Proliferation to MBP fragments in Lewis rats fed whole MBP orally.

<u>Source of LNC</u>	<u>Whole MBP</u>	<u>1-37</u>	<u>44-89</u>
SPC	5.10 \pm 1.6	5.05 \pm 1.8	2.41 \pm 0.9
Mes.LNC	8.61 \pm 1.9	9.88 \pm 1.5	3.53 \pm 0.8
Cervicals	4.58 \pm 1.3	6.42 \pm 0.9	2.51 \pm 0.6

Animals were fed 1 mg of whole MBP x3, then cells removed from various organs 15 days following feeding and proliferation measured. Results are expressed as the change in CPM $\times 10^{-3}$ as compared to cells cultured

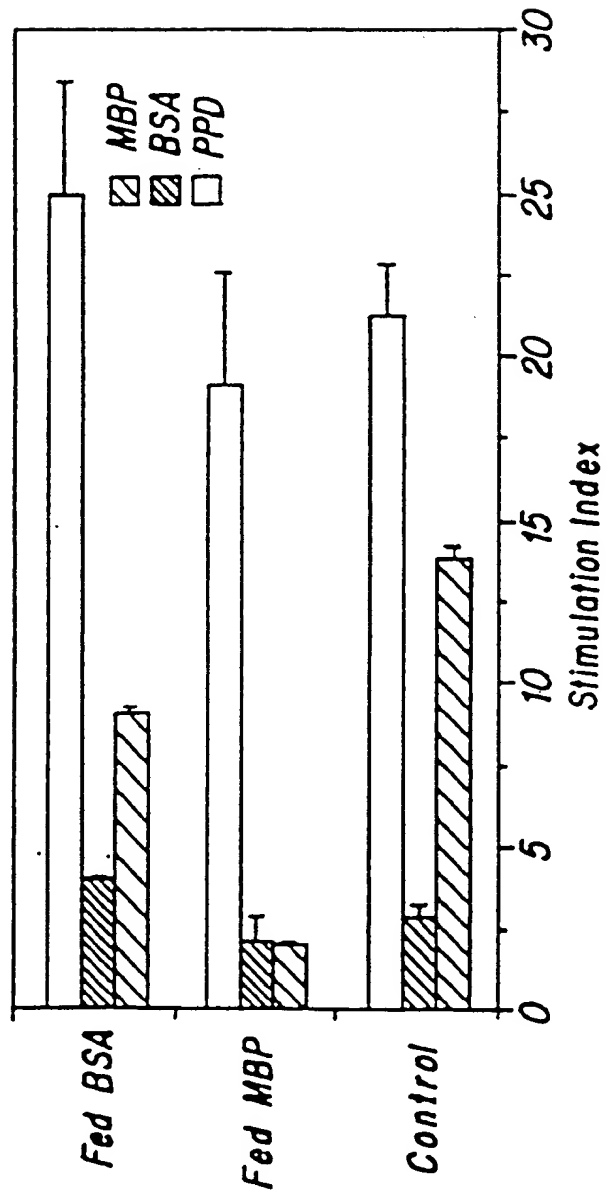


FIG. 1

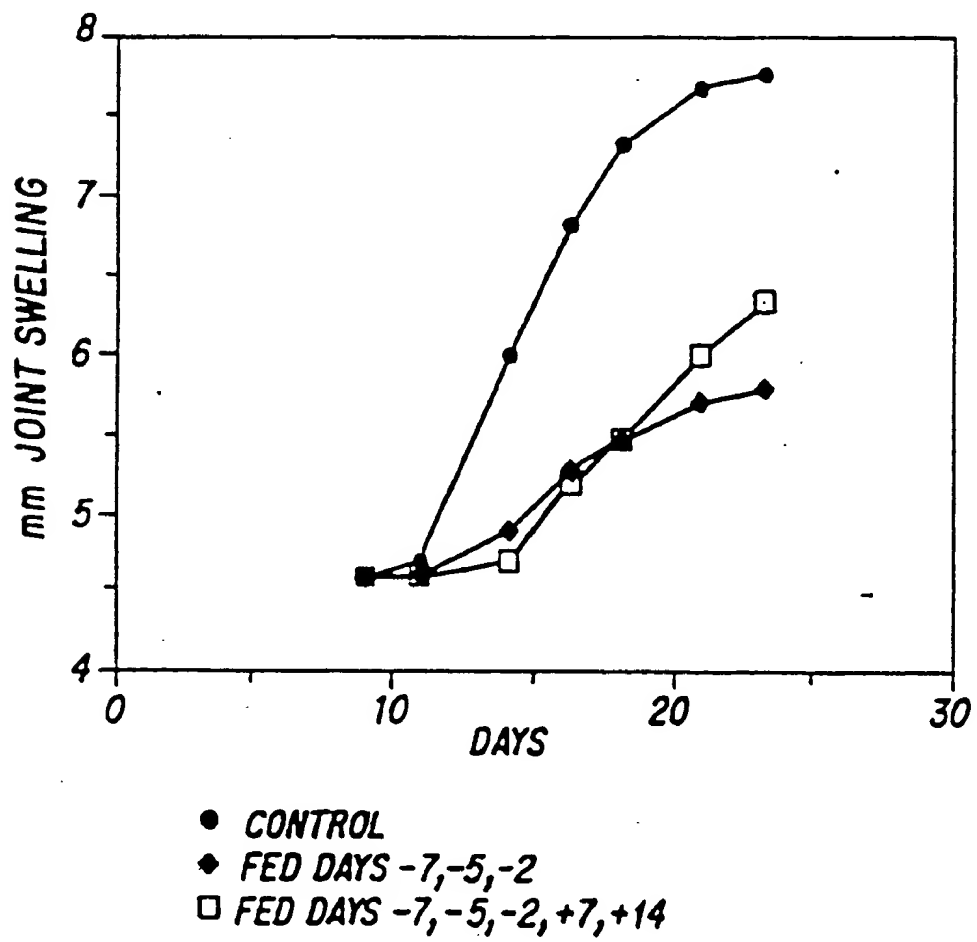


FIG. 2

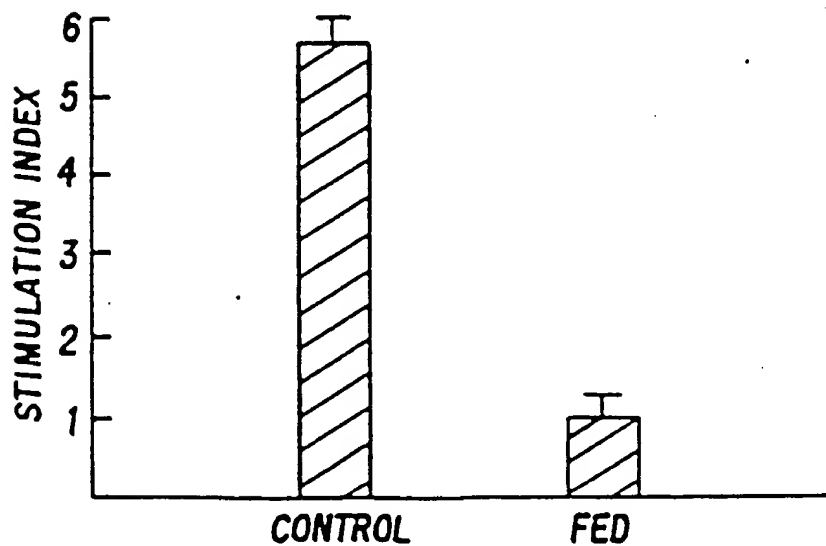
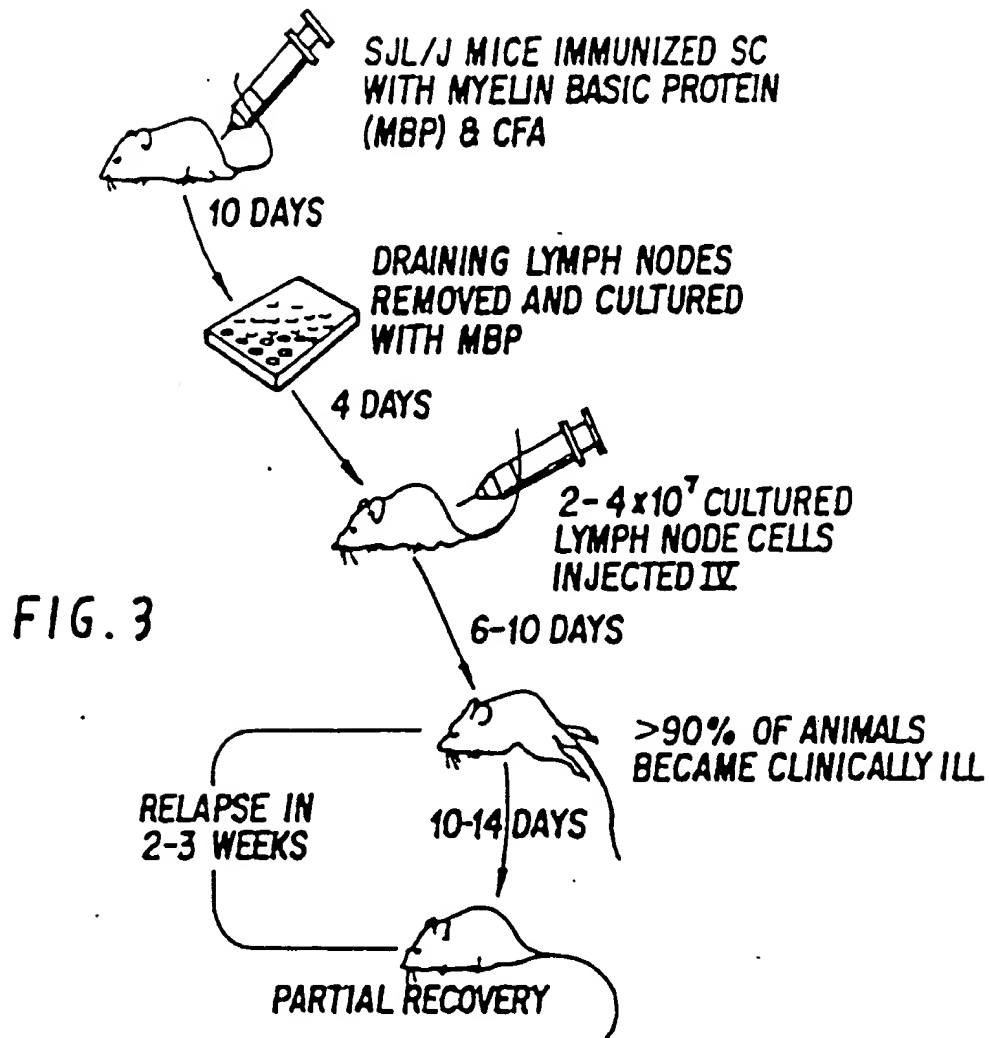


FIG 4

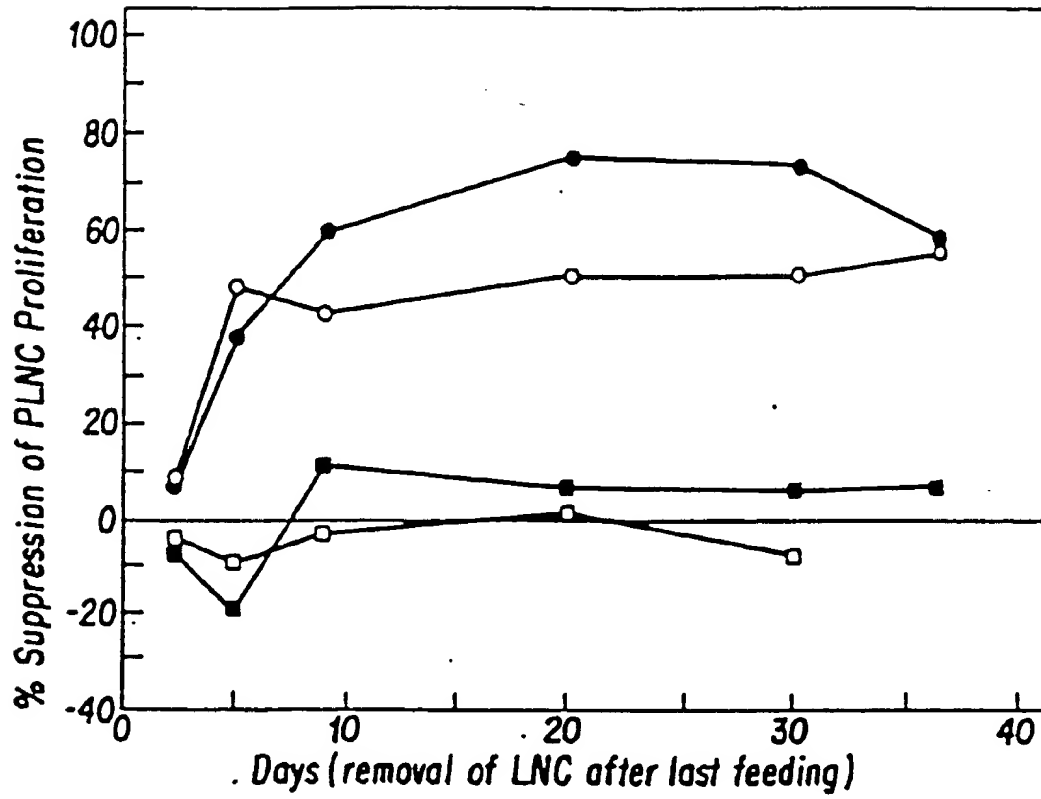


FIG. 5

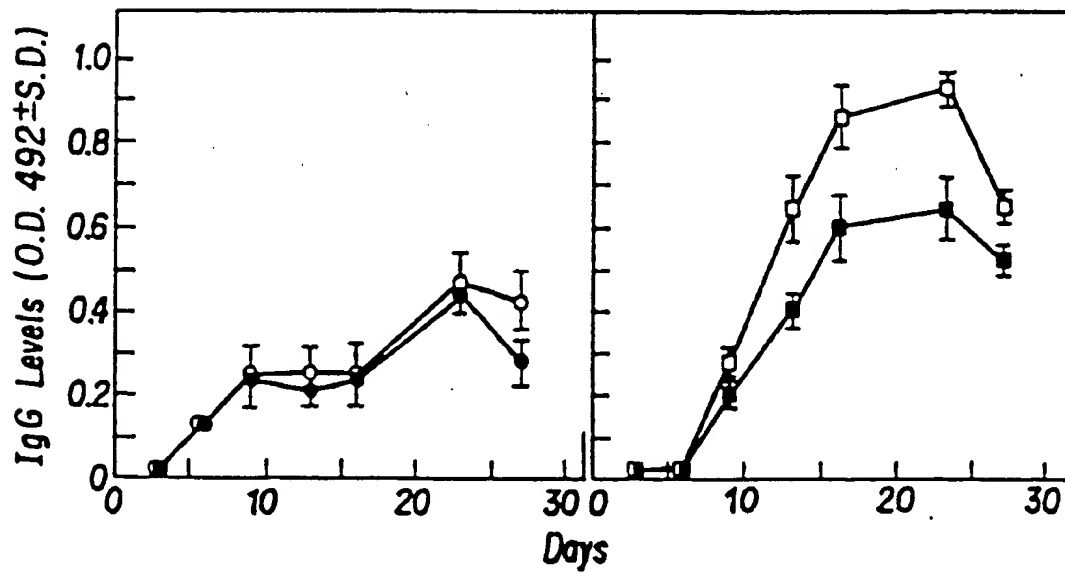


FIG. 6



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Application Number
EP 95 10 2528

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.4)
O,X	CELLULAR IMMUNOLOGY, vol.112, no.2, 1 April 1988 pages 364 - 370 DINA M. BITAR ET AL. 'SUPPRESSION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS BY THE ORAL ADMINISTRATION OF MYELIN BASIC PROTEIN (1).' (1) THIS WORK HAS BEEN PRESENTED IN PART AT THE SIXTH INTERNATIONAL CONGRESS OF IMMUNOLOGY, TORONTO, CANADA, JULY 7-11, 1986." * the whole document *	1	A61K39/00
X,D	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol.83, October 1986, WASHINGTON US pages 7443 - 7446 CATHRYN NAGLER-ANDERSON ET AL. 'SUPPRESSION OF TYPE II COLLAGEN-INDUCED ARTHRITIS BY INTRAGASTRIC ADMINISTRATION OF SOLUBLE TYPE II COLLAGEN.' * the whole document *	1	TECHNICAL FIELDS SEARCHED (Int.Cl.4)
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The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 12 May 1995	Examiner Rempp, G
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding</p>			



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Application Number
EP 95 10 2528

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.4)
X	ANNALS OF NEUROLOGY, vol.20, no.1, July 1986 pages 161 - 162 PAUL J. HIGGINS ET AL. 'SUPPRESSION OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS BY THE ORAL ADMINISTRATION OF MYELIN BASIC PROTEIN.' ABSTRACT P154	1	
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X	US-A-3 989 819 (JOHN A. BROCKMAN) * column 1, line 11 - column 3, line 68 *	1	
X,P	JOURNAL OF IMMUNOLOGY, vol.140, no.2, 15 January 1988, BALTIMORE US pages 440 - 445 PAUL J. HIGGINS ET AL. 'SUPPRESSION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS BY ORAL ADMINISTRATION OF MYELIN BASIC PROTEIN AND ITS FRAGMENTS (1).' * the whole document *	1	
A	ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY, vol.98, 1978 pages 259 - 281 E. H. EYLAR 'PEPTIDES AND AUTOIMMUNE DISEASE.'		TECHNICAL FIELDS SEARCHED (Int.Cl.4)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 12 May 1995	Examiner Rempp, G
CATEGORY OF CITED DOCUMENTS		I : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure & : member of the same patent family, corresponding	